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<b>(21) International Application Number:</b> PCT/US88/00762 <b>(22) International Filing Date:</b> 11 March 1988 (11.03.88)  <b>(31) Priority Application Numbers:</b> 025,303 130,911 <b>(32) Priority Dates:</b> 13 March 1987 (13.03.87) 10 December 1987 (10.12.87) <b>(33) Priority Country:</b> US  <b>(71) Applicant:</b> COULTER ELECTRONICS, INC. [US/ US]; 590 W. 20th Street, Hialeah, FL 33010 (US). <b>(72) Inventors:</b> LEDIS, Stephen, L. ; 759 E. 56th Street, Hialeah, FL 33010 (US). CREWS, Harold, R. ; 8960 N.W. 11th Street, Pembroke Pines, FL 33024 (US). FISCHER, Timothy, J. ; 1380 S.W. 82nd Terrace, Plantation, FL 33324 (US). SENA, Ted ; 1401 N.E. 123rd Street, Miami, FL 33161 (US).		<b>(74) Agent:</b> HIBNICK, Gerald, R.; Coulter Electronics, Inc., 590 West 20th Street, Hialeah, FL 33010 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> SYSTEM FOR ISOLATING AND IDENTIFYING LEUKOCYTES  <b>(57) Abstract</b>  A method and reagent system for the rapid isolation, identification, and/or analysis of leukocytes from a whole blood sample. The reagent system includes a lytic reagent solution comprising an aqueous solution of a water soluble compound which at least partially dissociates in aqueous solution to release a proton and a counterion and which, upon contact with a blood sample, acidifies the sample to a pH in the range of from about 2.6 to 4.0. The reagent system also includes a quenching reagent solution comprising an aqueous alkaline salt solution which effectively and rapidly arrests the chemical action of the lytic reagent solution on the sample and restores the native physiological environment of the sample. The method comprises using the reagent system to selectively lyse the red blood cells in a blood sample and modify the leukocytes to enable their differentiation into five sub-populations.		

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## SYSTEM FOR ISOLATING AND IDENTIFYING LEUKOCYTES

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BACKGROUND OF THE INVENTION

Field of the Invention This invention is directed to compositions  
10 of matter and methods employing one or more of these  
compositions. More specifically, this invention concerns a novel  
lytic reagent system for rapidly effecting hemolysis of the  
erythrocyte fraction of whole blood and thereby permitting the  
isolation of the leukocyte fraction in its native or near native  
15 state. The leukocyte fraction may then be subjected to further  
study or analysis in a variety of environments. The lytic reagent  
of this invention consists essentially of a water soluble compound  
which at least partially dissociates in aqueous media, thereby  
releasing a proton and a counterion. When the appropriate  
20 concentration of such compound is added to a whole blood  
sample, the extent of dissociation is effective to acidify the  
sample (pH in the range of from 2.6 to about 4.0), while  
maintaining the osmolality of the sample below about 100 mOs.  
One of the preferred uses of this lytic reagent system is the  
25 pretreatment of the whole blood sample to effect rapid and  
essentially complete hemolysis of the erythrocyte fraction. Such  
pretreatment also results in subtle modification to the leukocyte  
fraction, thus, facilitating its further differentiation into at least  
five (5) distinct sub-populations. This reagent system is, thus,  
30 suitable for preparation of a whole blood sample for analysis on a  
focused flow analysis system, such as the VCS whole blood  
analyzer and the Epics Model C and PROFILE flow cytometer (all  
of which are available from Coulter Electronics, Inc., Hialeah,  
Florida.

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Description of the Prior Art - The separation of complex biological fluids (i.e. whole blood) into its various constituents preliminary to study and analysis of its component parts, is generally desirable and often an essential requirement of many established analytical protocols and/or instrumentation utilized in such studies and analysis. Where such study or analysis of the fluid fraction of the sample is of primary interest, the cellular fraction is separated from the sample without regard for maintaining the cell viability or membrane integrity. Conversely, where the cellular fraction is itself of primary interest to the researcher or clinician, the partitioning of the whole blood sample into its various cellular components requires that the sample treatment/processing techniques be adjusted accordingly. The traditional methods for separation of whole blood samples into its various cellular components is by centrifugation. While this process is effective, it is labor intensive, relatively inefficient and requires physical manipulation of the cellular fraction of the sample.

Where such separation/partitioning of the cellular fraction of the sample is attempted with chemical agents, the results have been less than totally satisfactory for a variety of reasons. More specifically, the vitality and viability of a cell population in vivo or in vitro is dependent upon maintaining a precise physiological environment that is consistent with the preservation of physical cell structure and chemical balance within the cell. This balance is controlled by the permeability and transport characteristics of the cell membrane. Alteration in the physiological environment will in turn evoke a response or change in the cell membrane. The membrane response is "defensive" in nature; that is, the physiological response of the membrane is calculated to maintain the chemical balance within the cell and, thus, its continued and uninterrupted vitality.

It is fully appreciated that such alteration in the ideal physiological environment of the cell can be tolerated only within limits; and, that when such limits are exceeded, permanent injury to the cell can occur. As is further appreciated in the art, such changes in environment (i.e. toxicity of the diluting medium - even with distilled water) can effect hemolysis of the cells.

10 The degree of tolerance of various cell populations in whole blood to changes in their physiological environment has been extensively studied and documented. The effects of alteration in various aspects of the physiological environment of the cellular fraction of whole blood are both subtle and dramatic and can be  
15 effected through dietary metabolites and/or foreign substances. These studies include monitoring the reaction of cellular preparations to different drugs, Da Costa, A.J. et al, Transfusion, (1973), 13, 305; to dietary imbalance, Kobayashi, T. et al, Journal of Biochemistry (1983) 93, 675; and to changes in pH, Rother, U.  
20 et al, Z. Immunologie Forschungsgemeinschaft (1978) 155, 118, and Schettini, F. et al, Acta Paediat. Scand. (1971), 60, 17.

In each of the articles noted above, a drug, food metabolite or  
25 change in pH resulted in significant alteration in the physiological environment of the blood to the degree where hemolysis of the red blood cells was effected.

30 More specifically, the above referenced Rother article reports serum activation by acidification (pH 6.4) with hydrochloric acid which lysed unsensitized erythrocytes in the presence of EDTA. The article compares the effect of such acidification with the "deviated lysis" activity observed following serum activation with  
35 insulin. An independent and unrelated study by Schettini and his

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co-workers concluded that red blood cells from infants and young children were more sensitive to acid hemolysis than red blood cells from older individuals.

5 As of the present, no chemical treatment is available to rapidly and effectively partition a whole blood sample into viable cellular fractions. Where one or more chemical treatments of the whole blood sample is used (as in the preparation or pretreatment of  
10 whole blood for the performance of white blood cell differentials), the focus of such treatment has been to alter the sample to permit the differentiation of its cellular components from one another based upon isolation/analysis of (i) the cellular debris (i.e. nuclei) remaining subsequent to such treatment; (ii) the  
15 fixation of the cells by such chemical treatments; or (iii) the relatively severe modification of such cells which eventually results in their ultimate disintegration. Such relatively harsh and disruptive chemical treatments of whole blood samples has, however, been successfully applied where combined with  
20 relatively sophisticated instrumentation. More specifically, the ability to alter the physiological environment of a cellular population in vitro has been used to advantage in the measurement of certain cellular parameters and to quantitate the individual populations. The distinctive reaction of each individual  
25 cell population to a change in its physiological environment has particular advantages in the identification of the individual leukocyte sub-populations of cells of whole blood. The leukocyte population of blood has been classified previously into two major fractions: the lymphoid and the myeloid fraction. The lymphoid  
30 fraction consists of lymphocytes (B and T cells). The myeloid fraction consists of monocytes and granulocytes (neutrophils, basophils and eosinophils). One accepted technique for modification of the physiological environment of the cellular population of whole blood has been through the addition of  
35 certain so-called "lytic reagents" to a blood sample. The

development of certain lytic reagents and lytic reagent systems has provided the clinician with the ability to effectively isolate the white cell population (hereinafter "leukocytes") from the red cell population of whole blood. The relative concentration of leukocytes within the blood sample and the gross morphological appearance of certain classes of these cells can be clinically significant.

10 This ability to further differentiate the leukocyte population, thus, provides an invaluable diagnostic tool in the study and treatment of various diseases. As is further appreciated, the larger the number of sub-populations of leukocytes which are identifiable, the more accurate and reliable the identification of  
15 any one such sub-population.

A number of references have appeared in the recent patent literature which disclose various reagent systems and techniques  
20 for enhancing the ability of automated instrumentation to conduct white blood cell differentials. The following references are representative of the pertinent patent literature in this field: U.S. Patents 3,874,852; 4,286,963; 4,346,018; 4,485,175; 4,520,274; 4,529,704; and, U.S. Applications Serial No. 615,961  
25 (corresponding International Application PCT/US85/00954, published December 19, 1985); and, Serial No. 615,966 (corresponding International Application PCT/US85/00868, published December 19, 1985), all of which are hereby  
30 incorporated by reference in their entirety.

U.S. Patent 3,874,852 (to Hamill) describes a reagent system and method useful in the performance of leukocyte and hemoglobin determinations of whole blood. This reagent system comprises an  
35 essentially ferrocyanide free aqueous solution of quaternary

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ammonium salt and cyanide ions. This reagent system is effective to stromatolyze both red blood cells and platelet cells in whole blood and in the conversion of the free hemoglobin to a chromagen. This system is reported efficacious for leukocyte and hemoglobin determinations with diagnostic accuracy. The leukocyte population profile available with this system is, however, limited to total white cell count, without further differentiation of this cellular fraction into its discrete sub-populations.

10

U.S. Patent 4,286,963 (to Ledis, et al) describes a lytic diluent and method for achieving rapid lysis of red blood cells in whole blood. This diluent enhances the ability of automated instrumentation to perform differential determinations of lymphoid and myeloid sub-populations of leukocytes and the quantitative determination of hemoglobin. The lytic diluent described by Ledis is composed of a mixture of at least one quaternary ammonium salt and an aryl substituted short chain alkanol in buffered aqueous medium (pH 3.5 to 5.0). The lytic diluent of this Ledis patent is, however, limited in its ability to effect differentiation of the leukocyte population into the two (2) principle sub-populations; namely, the lymphoid and myeloid fractions.

25

U.S. Patent 4,346,018 (to Carter, et al) describes a multipurpose blood diluent and a method for utilizing this diluent in combination with a weak lysing reagent system for the performance of hemoglobin determination and the differentiation of lymphocytes into the lymphoid and myeloid sub-populations. This diluent comprises, among other constituents, N-(2-acetamido)iminodiacetic acid (ADA) as a blood stabilizing agent. The lysing agent comprises an aqueous solution of at least one quaternary ammonium salt. The diluent/lytic reagent of this Carter patent is, however, limited in its ability to effect

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differentiation of the leukocyte population into the two (2) principle sub-populations; namely, the lymphoid and the myeloid fractions. In addition, ADA has been found to help stabilize the size distribution, cellular shape, and most importantly, the high degree of cellular dispersion of erythrocytes and platelets to an extent not previously observed with other compounds.

- 10 U.S. Patent 4,485,175 (to Ledis, et al) describes a reagent system and method for performance of differential determinations of leukocytes into three (3) sub-populations utilizing automated cell counting equipment. This reagent system comprises a blood diluent and a lysing agent. The lysing agent (comprising an aqueous mixture of quaternary ammonium salts), when added to
- 15 the diluted blood sample under mild conditions of concentration and at a relatively slow rate, causes unexpected volume modifications to the various leukocyte sub-populations. The discovery which permitted the attainment of the degree of differentiation of the leukocyte population by Ledis, et al is based
- 20 upon the observation of the relative greater sensitivity of the granulocyte sub-population to lytic agents. By controlling the rate of exposure of the lymphocyte population to lytic agents, the granulocyte sub-population is better preserved. The reagent system of this Ledis et al patent is, however, limited in its ability
- 25 to effect differentiation of the leukocyte population into three (3) sub-populations; namely, lymphocytes, monocytes and granulocytes.
- 30 While all of the above lytic agents and reagent systems facilitate the differentiation of the leukocyte fraction of a blood sample (to a greater or lesser degree), each suffers from a common deficiency; namely, the inability to effect such differentiation without adversely altering the chemical balance of the cells which
- 35 are subjected to such treatment. Where such alteration in the

chemical balance is induced, the effect on the cellular population can range from relatively minor changes (i.e. swelling) to lysis. Dramatic chemical changes in the physiological environment of the leukocyte population also alters the immunochemical response of the leukocyte surface markers. The treatment of leukocytes with such traditional lytic agent system is, thus, inherently incompatible with further immunochemical study of these leukocytes. This limitation has thus, up to now, prevented the use of lytic reagents, alone or in combination with other means, for further refinement in the diagnostic process of various disease states, based upon the differences in the immunochemical response of the respective surface markers of each such cell population.

#### OBJECTS OF THE INVENTION

Accordingly, it is the object of this invention to remedy the above as well as related deficiencies in the prior art.

More specifically, it is the principle object of this invention to provide a chemical treatment, or pretreatment, of a complex biological fluid sample, such as whole blood, which facilitates the subsequent isolation, identification and/or analysis of one or more cellular populations that are present in the fluid sample.

It is another object of this invention to provide a chemical treatment, or pretreatment, of a whole blood sample which facilitates the partitioning of the sample and thereby the subsequent isolation, identification and/or analysis of the leukocyte fraction based upon the physical, physiological and/or

immunochemical properties of such fraction in its native or near native state.

- 5 It is yet another object of this invention to provide a chemical treatment, or pretreatment of a whole blood sample which is selective for only one of the cellular constituents of sample.
- 10 It is still yet another object of this invention to provide a reagent system which can rapidly and efficiently partition a whole blood sample into an essentially intact leukocyte fraction and a lysed erythrocyte fraction.
- 15 It is an additional object of this invention to provide a lytic reagent system for use in differential determination of leukocyte sub-populations of whole blood.
- 20 It is another additional object of this invention to provide a novel lytic reagent system which includes both a lytic reagent and a companion quench for use in differential determination of leukocyte sub-populations in whole blood.
- 25 It is yet another additional object of this invention to provide a novel lytic reagent system which is effective for use in differential determination of leukocyte sub-populations in whole blood by (a) measurement of physical and/or optical properties of such sub-populations, and/or (b) observation of the immunochemical response or interaction of such sub-populations with immunoreagents (i.e. antiserum) specific for one or more surface markers on each such cell sub-populations.

It is a further object of this invention to provide a method for performance of differential determination of leukocyte sub-populations by both (a) measurement of their physical and/or optical properties, and (b) immunochemical response of such sub-populations to antiserum specific for one or more surface markers on each said sub-populations.

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### SUMMARY OF THE INVENTION

The above and related objects are achieved by providing a chemical reagent system which is selective in its interactions with the various cellular components of a complex biological fluid sample. This reagent system will, in the various environments contemplated for its use, provide at least one reagent component to effectively alter, on a selective basis, one or more cellular constituents of the complex biological fluid sample to the degree necessary to permit the subsequent isolation, identification and/or analysis of the cellular constituents of interest. This reagent system further contemplates the ability to modulate the chemical treatment of the cellular constituents of the sample by providing a separate reagent which is designed to quench or retard the action of the cell altering reagent on the cellular populations of the sample.

The principles and concepts of this invention have been successfully applied to the treatment of whole blood samples preliminary to white blood cell differential analysis. The basic components of the chemical reagent system of this invention include a "lytic reagent" and a companion reagent for the lytic reagent referred to as a "quench." The primary functions of the quench are to retard the activity of the lytic reagent and to

restore the ionic balance of the sample subsequent to its treatment with the lytic reagent.

5 The lytic reagent system and method of this invention, thus, has as its broadly stated objectives, the selective hemolysis of the erythrocyte population of a whole blood sample, while facilitating the subsequent isolation, identification and/or analysis of one or more of the leukocyte sub-populations of the same sample, based  
10 upon one or more physical, physiological and/or immunochemical characteristics which are indicative of the sub-population of interest. The lytic reagent of this invention consists essentially of a water soluble compound which at least partially dissociates in the aqueous media, thereby releasing a proton and a counterion.  
15 When the appropriate concentration of such compound is added to a whole blood sample, the extent of dissociation of such compound is effective to acidify the sample (pH in the range of from 2.6 to about 4.0), while maintaining the osmolality of the sample below about 100 mOs. There are at least three (3) classes  
20 of compounds which have been found suitable as lytic reagents for achieving the objectives of this invention. These classes include low molecular weight carboxylic acids, sulfonic acids and activated phenols.

25

The carboxylic acids suitable as lytic reagents of this invention can be represented by the following formula



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wherein R is H, an aliphatic hydrocarbon radical having from 1-3 carbon atoms; a carbonyl substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; a hydroxy substituted aliphatic hydrocarbon radical having  
35 from 1-3 carbon atoms; or an aliphatic hydrocarbon

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radical having from 1-3 carbon atoms and multiple carbonyl and/or hydroxy substituents.

5 Representative carboxylic acids within the foregoing formula include formic acid, methane-carboxylic acid, (acetic acid), 2-hydroxy-ethane-2-carboxylic acid (lactic acid); 1,2-ethane-dicarboxylic acid (succinic acid); and 2-hydroxy-1,2,3-propane-tricarboxylic acid (citric acid); and their respective mixtures.

10

The sulfonic acids suitable as lytic reagents of this invention can be represented by the following formula

15



wherein R is hydroxy, an aliphatic hydrocarbon radical of 1-3 carbon atoms or aryl.

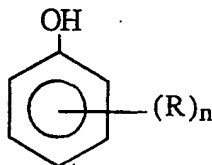
20

Representative sulfonic acids within the foregoing formula include sulfuric acid; methanesulfonic acid; ethanesulfonic acid; benzenesulfonic acid; p-toluenesulfonic acid; nitrobenzenesulfonic acid, and their respective mixtures.

25

The activated phenols suitable as lytic reagents of this invention can be represented by the following formula

30



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wherein R is an electron withdrawing group such as halogen, cyano, nitro or any combination of such electron withdrawing substituents; and n is 1-3.

5

Representative activated phenols within the foregoing formula include para-nitrophenol; meta-nitrophenol; ortho-nitrophenol; 2,4-dinitrophenol; para-chlorophenol; para-cyanophenol; and 1-chloro-2,4-dinitrophenol; and their respective mixtures.

10

The foregoing are representative of the classes of materials which can be used as lytic reagents consistent with the objectives of this invention. It is appreciated that the relatively weak acids will  
15 exist as both an undissociated compound and in the dissociated state within the sample. Strong acids are, of course, essentially completely dissociated in the sample. Certain undissociated acid and the counterions from the dissociated acids can apparently influence the degree of differentiation of the leukocyte fraction,  
20 depending upon their relative concentration in the sample and the physiological recognition (if any) of the acid and/or its counterion by the cellular analytes of interest. For example, phosphoric acid, even at the appropriate pH, is generally not acceptable to achieve differentiation of the leukocyte into five (5)  
25 sub-populations. It is hypothesized that counterion compatibility (phosphate ion) is unacceptable and, thus, differentiation of the leukocyte population is decidedly more difficult.

30

In this preferred embodiment of the concepts of this invention, the novel lytic reagent system comprises an aqueous solution containing a differentiation effective amount of lytic reagent selected from the group consisting of formic acid, acetic acid and  
35 their respective mixtures. In these mixtures, the formic acid will

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preferably comprise the major functional component with the acetic acid being present in only minor amounts. The phrase "differentiation effective amount" is used throughout this disclosure as indicative of a concentration of lytic reagent which

5 is not only effective for lysing red blood cells, but also effects subtle changes in the leukocyte cell fraction to facilitate the subsequent isolation, identification and/or analysis of this leukocyte cellular fraction; including the ability of instrumentation to perform differential analysis and

10 identification of at least five (5) sub-populations of leukocytes. The preferred range of concentration of formic acid in the lytic reagent is in the range of from about 0.10 to about 0.25% (v/v). The concentration of such preferred lytic reagent which has been determined as satisfying the foregoing criteria is from about .009

15 to about 0.020 milliliters of formic acid per milliliter of whole blood. This subtle modification of the leukocyte fraction by the lytic reagent is achieved while preserving the immunochemical response of the surface markers of each of the five (5) cell sub-populations of leukocytes. As stated previously, it is the primary

20 objective in this treatment of the whole blood sample with the lytic reagent, that such reagent effectively accomplishes stromatolysis of the erythrocyte cell fraction while preserving the leukocyte fraction in its essentially native state.

25

In one of the preferred embodiments of this invention, the reagent system can contain saponin in addition to the lytic reagent. The term "saponin" is intended as referring to commercial grade quillaja saponin powder. The addition of

30 saponin to the reagent system is optional and generally only appropriate where the clinician is monitoring certain parameters of the leukocyte sub-populations other than by photooptical or immunochemical techniques. The addition of appropriate quantities of saponin to the lytic reagent system is effective in its

35 ability to reduce the size of red cell fragments so as to prevent



their interference in determination of certain leukocyte parameters by measurement of electrical opacity and/or Coulter volume utilizing the techniques described in Coulter U.S. Patents 2,656,508 and 3,502,974 (which are hereby incorporated by reference in their entirety). The preferred range of concentration of saponin which has been determined as effective for reducing red cell fragments is from about 0.006 to about 0.012 grams per milliliter of whole blood. In brief, the technique involves the measurement of cell volume utilizing radio frequency current (RF) and DC field excitation. By generating a particle sensing field with both a low frequency or direct current (DC) and radio frequency (RF) current excitations, two or more interrelated output signals can be derived from passage of a single particle (i.e. leukocyte) through an electric field. The value derived from this output signal is termed "relative opacity", which is unique for each sub-population of leukocyte. The addition of saponin to the lytic reagent reduces the size of the red blood fragments to a point where they will not interfere with or themselves cause, the derivation of an output signal indicative of a species of leukocyte.

20

Where cell differentiation is based upon light scatter measurements, (utilizing the techniques described in Fulwyler U.S. Patent 3,989,381 and/or Auer, et al U.S. Patent 4,038,556 - which are hereby incorporated by reference in their entirety), the red cell fragments do not interfere or adversely effect the photometric differentiation of the various sub-populations of leukocytes. Thus, the addition of saponin to the lytic reagent system is unnecessary where such differentiation is based upon photometric analysis.

30

The amount of time of exposure of the blood sample to the lytic reagent system is critical to the differentiation method of this invention. This exposure period, as illustrated in the Examples

35

disclosed hereinafter, should not exceed ten (10) seconds, and most preferably, requires only about six (6) seconds or less. Both of these exposure times are specified for room temperature ( $\sim 18-28^{\circ}\text{C}$ ). In each instance, the action of the lytic reagent is

5 quenched by simple addition of appropriate concentrations of salts to the sample to return the cells to their native physiological environment. The quench effectively retards further activity of the lytic reagent upon the leukocytes without need for the addition of fixatives. The quench for the lytic reagent is an

10 essential complement to the lytic reagent system where subsequent analysis of the leukocyte fraction requires retarding the activity of the lytic reagent. The leukocytes are stabilized by this quench by controlling the pH within a fairly narrow range (pH  $\sim 6.00$  to  $7.25$ ) and osmolality ( $\sim 300$  to  $330$  milliosmoles). The

15 quench can also be formulated to match the conductivity of a chosen "sheath" fluid which is utilized in a focused flow aperture analysis system. The composition and volume of the quench is adjusted to provide optimal separation of the five (5) major leukocyte subclasses when analyzed in accordance with the

20 techniques (RF frequency current in combination with DC field excitation) described in Coulter, et al U.S. Patent 3,502,974 (previously incorporated by reference). The leukocyte fraction of the sample, treated in the above manner, can be readily differentiated to at least five (5) sub-populations by a

25 hematology analyzer capable of multiple parameter particle (cell) measurements; and, by immunochemical interaction with antiserum (i.e. antibodies, binding proteins, etc.) that are specific for one or more surface markers on the cells of each such cell sub-population.

### BRIEF DESCRIPTION OF THE DRAWINGS

- 5 Figs. 1, 2 and 3 are scattergrams representing the white cell differential analysis of the blood sample of Example II; the X-axis of each such Fig. being different from one another.
- 10 Fig. 4 is a scattergram representing the white cell differential analysis of the blood sample of Example I.
- Fig. 5 is a scattergram representing the white cell differential  
15 analysis of the blood sample of Example V.
- Fig. 6 is a scattergram representing the white cell differential  
20 analysis of the blood sample of Example VI.

### DESCRIPTION OF THE INVENTION INCLUDING PREFERRED EMBODIMENTS

- 25 The lytic reagent system of this invention comprises an aqueous solution containing surprisingly low concentrations of the lytic reagent (preferably less than 1.0% by volume). The lytic reagent of the reagent system of this invention can generally be described  
30 as a water soluble, acidic compound which at least partially dissociates in aqueous media. As noted above in the Summary of the Invention, a number of interrelated factors are believed to be essential for such compounds to perform within the various environments contemplated for this invention. These factors  
35 include pH, osmolality and counterion compatibility. The

acidification of the sample is necessary to effect the desired lysis of the erythrocytes. Where, however, the pH of this sample departs from the preferred range of from about 2.6 to about 4.0, the ability to differentiate the leukocyte fraction into five (5) distinct subpopulations is apparently compromised.

This ability to effectively differentiate the leukocyte population into its various subpopulations is also hindered where the osmolality of the lysed sample is not maintained below about 100 mOs. Accordingly, where either pH or osmolality of the lysed sample is not maintained within the foregoing parameters, the ability to effectively differentiate between the various subpopulations of leukocytes is significantly impaired.

The counterion of the dissociated acidic compound has, in certain instances, been shown to effect the degree to which differentiation of the leukocyte fraction can be effected. For example, phosphoric acid, even at concentrations which produce the appropriate pH and osmolality, is not (as) effective in the differentiation of this leukocyte fraction into its various subpopulations. It is hypothesized that the counterion of this acid (phosphate) interacts in some unknown manner or fails to interact with the leukocyte fraction, and thereby does not afford the extent of differentiation afforded by other water soluble, acidic compounds.

There may also be other factors and mechanisms which are inherent in the action of the lytic reagents upon the sample which account for the surprising and unexpected results of this invention. As of the present, only the above three (3) enumerated variables have been identified; and it is not intended to imply that there may not be others or that this invention is dependant upon only the above identified variables for its effectiveness.

The lytic reagent of this invention is preferably an aqueous solution of formic acid, acetic acid or mixtures of formic and acetic acid in which formic acid is the predominant functional component. This aqueous solution is prepared by simple addition of the lytic reagent to deionized water. The amount of lytic reagent added to this diluent is sufficient to prepare a solution containing from about 0.05 to about 0.5% (v/v) solution. In the preferred embodiments of this invention, the concentration of lytic reagent will range from about 0.1 to about 0.25% (v/v).

Where the lytic reagent comprises a mixture containing both formic and acetic acid, the acetic acid is preferably only present as a partial replacement for a definitive amount of formic acid and then only at a concentration in the range of from about 0.05 to 0.10% (v/v).

The lytic reagent system also optionally can contain very small amounts of saponin (preferably at least about 0.05 up to about 0.2 weight percent) in addition to the lytic reagent. As is fully appreciated, the effectiveness of saponin, as a lytic reagent, is highly concentration dependent. If saponin is used at low concentrations, it is generally ineffective for this purpose. Were saponin to be used as a lytic reagent, it would have to be present in concentrations of at least 2.0 weight percent, or more, to effect essentially complete stromatolysis of the red blood cells. Unfortunately, the saponin, where present at a lytic effective concentration, can also cause lysis of white blood cells. Thus, any changes induced in the white blood cell population utilizing saponin as the lytic agent, and their subsequent differentiation by automated instrumentation must be based upon whatever discernible and distinctive characteristic of their respective nuclei. Its presence in the lytic reagent system of this invention

- is, however, desirable where the clinician is concerned with so-called "ghosts" (intact red cell membranes) interfering with differential measurements based upon distinctions in physical and/or electrical properties. As noted previously, saponin would not be a necessary addition to the lytic reagent system if the differential measurements were made by purely photooptical instrumentation and/or by immunochemical analysis.
- 10 The lytic reagent system can also contain other traditional additives, to the extent their presence is not otherwise incompatible with the primary functional components of the system (i.e. anti-microbial preservatives, such as sodium omadine.
- 15
- The lytic reagent system of this invention can be combined with a whole blood sample by simple manual or automated addition, the lytic reagent and sample allowed to briefly interact, and the action of the lytic reagent substantially retarded by addition of a suitable quench. The quench, to be effective in this environment, must, thus, be capable of retarding the lytic activity of the lytic reagent immediately upon its addition to the aqueous mixture contained in the blood sample and the lytic reagent. The precise formulation of the quench can vary, depending upon the composition of the lytic reagent system and the sheath fluid used in a focused flow aperture analysis system. The quench is typically an aqueous solution containing soluble salts which are both effective to substantially retard and/or substantially neutralize the lytic activity of the lytic reagent and restore the ionic balance to the sample. This restoration of the ionic balance will extend the longevity of the surviving cells and permit subsequent analysis on equipment which requires that the sample be electrically conductive (contain electrolytes), as for example with a Coulter Counter® whole blood analyzer.

A quench which is suitable for use in conjunction with a lytic reagent composition of this invention can, and usually will, contain any combination of at least two of the following four ingredients: sodium chloride, sodium sulfate, sodium bicarbonate, sodium carbonate; and, in addition, sodium azide as a preservative. The effectiveness of the quench upon the lytic reagent in the context of this invention is determined by its ability to rapidly reduce the lytic activity of the carboxylic and non-volatile mineral acids selected for use in the lytic reagent system. As noted above, the method and equipment utilized in the differentiation of the leukocyte sub-population can also place certain requirements (i.e. conductivity, pH, etc.) upon the precise formulation of the quench. More specifically, when such differentiation is performed in a focused flow aperture analysis system, the composition and volume of quench can be critical to optimal separation (differentiation) of the five (5) leukocyte subclasses from one another. In this type of analysis system, the ionic balance of the quench must also be adjusted to obtain a satisfactory conductivity match of the lysed blood sample to the sheath fluid. In the preferred quench formulation, the major ionic species and their relative ratio in the lysed-quenched blood sample should be essentially the same as the major ionic species and their relative ratio in the sheath fluid. The relative concentration of the functional components of the quench, which is to be used in conjunction with the lytic reagent system of this invention, will range from about 1 to about 3% (w/v) sodium chloride, about 0.25 to about 0.8% (w/v) sodium carbonate or bicarbonate, and about 2 to about 4% (w/v) sodium sulfate. The precise relative quantities of ingredients of the optimum quench are generally determined empirically; the objectives of such adjustment being to attain the pH of the lysed blood sample within the range of from about 6.0 to about 7.5, and a final osmolality of the stabilized lysed blood sample in the range of

from about 300 to about 330 mOs. It has been previously observed, in a focused flow aperture system, that optimal clustering of the leukocyte subclasses is achieved by adjustment in the osmolality of the final blood sample to about 310 mOs. The essentially complete neutralization of the acidity of the lysed sample with an alkaline quench can be critical to a focused flow aperture analysis system. Components of the sample (i.e. fibrin and platelets) are pH sensitive and can form aggregates under acidic conditions which can potentially interfere with differential analysis (i.e. noise) or physically obstruct the aperture of a focused flow aperture system.

It is not contemplated, nor intended, that the quench also necessarily inhibit or neutralize the saponin (when present). The reasons for this are quite simple, in that the saponin, at the concentrations contemplated (~0.2 weight percent), is relatively ineffective as a lytic reagent. If the quench were to also inhibit the saponin activity upon its addition to the sample, adequate clarification of the sample (destruction of intact red blood cell membranes) would not take place. Thus, it is anticipated that the activity of the saponin continue of from about 5 to 15 seconds subsequent to quenching of the activity of the lytic reagent. It may, under some circumstances, be appropriate to provide an independent quenching agent for the saponin; however, at the concentrations presently contemplated, (less than 0.1%) none would appear necessary to achieve or further the objectives of this invention. The adequacy of the quenching agent is, of course, based upon certain assumptions relating to sensitivity of cells of sample (analytes of interest) to the lytic reagent and the period of contact of the lytic reagent and these cellular analytes prior to analysis. As noted above, the quench retards the lytic activity, it does not totally eliminate its effect upon the leukocyte fraction of the sample. Thus, if a substantial period of time is to elapse between addition of quench and analysis of the sample, it may be



desirable to fix the leukocyte fraction to preserve the characteristic size and shape of the cellular analytes of interest.

- 5 In the preferred embodiments of this invention, the duration of effective contact of the lytic reagent and the blood sample (from the time the two are combined, to the time when the quench is added), must be less than ten (10) seconds, and most preferably six seconds or less. The interval of reactive contact of the lytic  
10 reagent and blood sample as specified above, presumes such reactive contact occurs at room temperature ( $\sim 18-28^{\circ}\text{C}$ ). Of course, if the temperature is in excess of this level, the period of reactive contact would be somewhat less and vary inversely; and, if the temperature is lower than this level, the period of reactive  
15 contact would be somewhat longer. It is both critical and essential to the successful performance of the differential method of this invention, that the kinetics of the interaction of the lytic reagent upon both the sacrificial cell population (red blood cells) and the desirable cell fraction (leukocytes) be controlled carefully  
20 and precisely. The mechanism by which the lytic reagent reacts with both cell fractions is not known with precision, only the manifest effect of such interaction. It is, thus, beyond the scope of this discussion to speculate how these improvements in differential analysis are attained and, thus, no attempt is made  
25 herein to explain or later claim such mechanism.

By limiting the exposure of both these cell fractions to the lytic reagent, stromatolysis of the erythrocytes is effectively and  
30 efficiently accomplished, while additional subtle changes are induced in the leukocytes by the quench to enable their effective differentiation. Both of these events occur essentially concurrently, while preserving the native immunochemical reactivity of the differentiated leukocyte fraction.

As noted above, the duration of contact of the lytic reagent with the blood is sufficient to effect a selective destructive response of the sacrificial cell fraction, while at the same time effecting a differential response in the leukocyte fraction; such changes unexpectedly permitting the physical differentiation of at least five (5) cell sub-populations of leukocytes from one another. The lytic reagent, in sharp contrast to the more traditional types of lyse (i.e. saponin, quaternary ammonium salts), does not detrimentally alter the native immunochemical response of the surface markers of each of the cells within the leukocyte sub-populations. This quality is believed to be unique to the lytic reagents of this invention.

Blood samples which have been exposed to the above lytic reagent system can be subjected to differential measurement on instrumentation designed for this purpose. Such differentials can be performed on a device utilizing technology of the type described in U.S. Patents 3,549,994 (which is hereby incorporated by reference in its entirety), 3,502,974 and 3,989,381. Features of the inventions described in the above referenced patents can be embodied into commercially practical instrumentation. In brief, the blood sample is initially treated by mixing with the lytic reagent system, utilizing automated pipetting equipment. The lytic action of the lytic reagent on the sacrificial cell population (red blood cells), must be both rapid and effective. A quench then is added by similar pipetting means to substantially retard the activity of the lytic reagent upon the surviving cell fraction (leukocyte). The sample containing the leukocyte fraction then is subjected to counting of each of the individual cell sub-populations, and/or histograms and/or scattergrams generated from the data collected in this fashion.

The principle involved in performance of Coulter volume measurements is well known to those skilled in the art as the "Coulter Principle." In brief, the operation of instrumentation utilizing the Coulter Principle involves the measurement of  
5 change in the impedance caused by the passage of individual cells through a sensor designed to detect a voltage drop caused by the presence of the cell. The instrumentation utilizing this principle comprises two fluid vessels or chambers, each containing a  
10 conductive electrolyte solution. At least two electrodes having opposite polarity are immersed in the electrolyte solution, with each fluid compartment having one of the electrodes disposed therein. A sample of the electrolyte solution, having the blood cells suspended therein, is passed through a constricted fluid path, or orifice, interposed between the two fluid compartments.  
15 Although the constricted pathway can take different forms, in each device such path defines a sensing zone wherein the presence or absence of a particle gives rise to a detectable change in electrical characteristics of the path. For example, relatively poorly conductive blood cells passing through this path, displace a  
20 volume of electrolyte solution equal to the cell volume, causing a voltage drop by increasing the path impedance. The resistance pulses defined by the drops in voltage are used for particle counting and particle volume determination. The Coulter principle is more fully described in U.S. Patent 2,656,508. This  
25 technique for sensing and identifying specific cell populations can be enhanced by a combination of Coulter principle measurements with radio frequency excitation of the cells within the sensing zone. In brief, this radio frequency enhancement operates upon the principle that a particle moving through the sensing zone of a  
30 hematology analyzer will cause a phase shift in radio frequency (RF) energy within the sensing zone. This shift in phase can be correlated with physical and compositional characteristics of a cell population. This technique for RF differentiation of cells is more fully described in U.S. Patent 3,502,974. White cell  
35 differentiation also can be achieved utilizing optical measurement

principles of flow cytometry as described in U.S. Patent 3,380,584 (which is hereby incorporated by reference in its entirety).

5 The lytic reagent system of this invention is effective to induce subtle changes in the leukocyte cell fraction to enhance the differentiation of five (5) distinct sub-population of leukocytes on automated cell counting equipment, see for example, U.S. 4,412,004 (to Ornstein, et al - which is hereby incorporated by  
10 reference in its entirety). These five (5) distinct sub-populations, as noted previously, include: lymphocytes, monocytes and three species of granulocytes (eosinophils, basophils and neutrophils). Each of these sub-populations of leukocytes have distinctive surface markers. In certain disease states, the surface markers  
15 on one or more of these sub-populations will provide a unique immunochemical response and, thus, make diagnosis or confirmation of disease possible at an early stage of its development. The detection of these distinctive surface markers on one or more of these sub-populations of leukocytes will, of  
20 course, be dependent upon the ability to effectively physically isolate these cells having characteristic disease state surface markers from the non-effected cells; and, the relative concentration of the effected cells within the sample being analyzed.

25

The initial lytic conditions contemplated by this invention permit an extension in the longevity of the cell populations which survive and which are to be differentiated from one another. For  
30 the most part, it is anticipated that cell longevity of at least seventy-two (72) hours would be appropriate for certain types of immunochemical analysis. In certain applications of this differential method, it may be both necessary and appropriate to maintain one or more of these sub-populations in vitro for  
35 several hours, or possibly even several days. In order to achieve

such extended stability, it is advisable to physically separate the leukocytes from the fluid fraction containing the lytic reagent/quench mixture and, thereafter, resuspended such cells in a physiological medium.

5

The following examples are provided as illustrative of the unique advantages of the lytic reagent system of this invention. The equipment and techniques utilized in the preparation and  
10 evaluation of this lytic reagent system are standard or as hereinbefore described. Parts and percentages appearing in such Examples are by weight unless otherwise stipulated.

15

#### EXAMPLE I

The lytic reagent system of this invention was prepared from reagent grade chemicals. A 0.12% (v/v) formic acid solution was  
20 initially prepared by combining 1.3 ml of 90% formic acid and 998 ml deionized water. A 50  $\mu$ L whole blood sample ( $K_3$  EDTA) and 800 microliters of 0.12% formic acid solution are gently mixed by swirling the two together for five (5) seconds at room temperature ( $\sim 20^\circ\text{C}$ ). The lytic action of the formic acid arrested  
25 after about 5 seconds, by the addition of 400  $\mu$ l of a quench solution containing 0.55% sodium bicarbonate, 3.0% sodium chloride and 0.01% sodium azide. The sample was adequately quenched and ready for differential analysis by flow cytometry techniques within about 5 to 10 seconds subsequent to the  
30 addition of the quench. The equipment used in such differential analysis was equipped with a helium/neon laser and silicon diode detectors for measurement of light scatter. The leukocytes are observed and their individual parameters determined by optical parameter analysis, i.e. measurement of light extinction (zero  
35 angle scatter), and any one of several angular ranges of light

scatter. The scattergram of the sample generated in the above manner is illustrated as Figure 4. Five (5) distinct sub-populations of leukocytes are identified and quantified in this scattergram.

5

### EXAMPLE II

- 10 The procedures of Example I are repeated, except for the addition of 0.05% (w/v) saponin powder to the aqueous solution containing 0.12% formic acid. The whole blood sample (50 microliters) is then combined (as previously described) with 600 microliters lytic reagent. The lytic reagent is quenched after 6 seconds by  
15 addition of 265 microliters of an aqueous solution containing 0.60% sodium carbonate, and 3.00% sodium chloride.

- The addition of saponin to the lytic composition effectively  
20 eliminates interference of the red cell debris from Coulter volume measurements. The effectiveness of the saponin is highly temperature dependent. The essentially total elimination of interference from red cell debris requires an additional 10 seconds (at room temperature ~18 to 28°C) subsequent to  
25 completion of lysing the red cell fraction of the sample. In the event the sample is maintained at a lower temperature (below 18°C), a somewhat longer period will be required to effectively eliminate interference from the red cell debris by the saponin.

30

- The sample was ready for differential analysis within about 10 to 20 seconds subsequent to the addition of the quench. The sample was subjected to photo-optical measurement as described in Example I. Leukocytes in the sample were also observed by  
35 measurement of DC and RF volumes using an ISOTON® II sheath

fluid and the resultant scattergram is illustrated in Fig. 1. Four distinct sub-populations of leukocytes were identified and quantified in the simultaneously obtained light scatter vs. DC scattergram of Fig. 2. A fifth sub-population of leukocyte (basophils) is isolated by generation of a "gated" secondary scattergram. This basophil population is depicted in the scattergram illustrated in Fig. 3.

10

### EXAMPLE III

The procedures of Example II are repeated, utilizing the same formic acid/saponin reagent composition. The lytic activity of the formic acid is arrested with a quench containing 3.13% (w/v) sodium sulfate (anhydrous), 1.45% (w/v) sodium chloride and 0.60% (w/v) sodium carbonate (anhydrous). The sample was analyzed by electro-optical techniques in the manner described in Example II and the scattergram results were similar to that of Figs. 1,2 and 3. The sheath fluid was, however, changed to ISOTON® III diluent.

25

### EXAMPLE IV

The procedures of Example III were repeated, except for use of a concentrated lysed-quenched blood sample for more rapid data acquisition. The lytic reagent comprises 0.15% (v/v) formic acid and contains 0.10% (w/v) saponin powder. The lytic activity of the formic acid was arrested by a quench containing 2.67% (w/v) sodium sulfate (anhydrous), 1.24% (w/v) sodium chloride and 0.56% (w/v) sodium carbonate (anhydrous).

35

This differential analysis was performed by addition of 50 microliters whole blood to a glass culture tube containing 300 microliters lyse reagent. The sample and lyse are mixed by swirling the contents of the tube for approximately 6 seconds and the lytic activity of the formic acid arrested by addition of 165 microliters of the above quench. The sample was then subjected to electro-optical measurement in the manner described in Example II and the resultant differential analysis was comparable to that of Example II, (as illustrated in Figs. 1,2 and 3).

10

#### EXAMPLE V

The procedures of Example I were repeated, except for the substitution of 0.1% (v/v) acetic acid for the formic acid in the lytic reagent system. The lytic activity of the reagent was arrested, after approximately 5 seconds, with a quench comprising 0.25% sodium bicarbonate in 2.0% sodium chloride solution. The sample was adequately quenched and ready for differential analysis by flow cytometry techniques within about 5 to 10 seconds subsequent to the addition of the quench. The equipment and analytical techniques used in such analysis were essentially the same as in Example I. The differential analysis performed on this sample is illustrated in the scattergram of Fig. 5.

25

5.

#### EXAMPLE VI

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The procedures of Example V were repeated except for extending the period of contact of the sample and the lytic reagent from 5 to 7 seconds prior to the addition of the quench. The quench was also modified slightly by increasing the concentration of sodium

35



bicarbonate from 0.25% to 0.375% in the 2.0% sodium chloride solution. The results of this differential analysis of the leukocyte population are illustrated in the scattergram of Fig. 6.

5

#### EXAMPLE VII

The procedures of Example I are repeated, except for the  
10 separation and identification of the various sub-populations of  
leukocyte by immunochemical techniques. Once the action of the  
lytic reagent is quenched, the sample is diluted with an isotonic  
buffer and sequentially slurried with a series of magnetic  
15 particles, each of which having been pre-treated with a different  
antiserum specific for adsorption of only one species of  
leukocytes. The adsorbed cells can be separated sequentially  
from the sample using the conventional magnetic particle  
separation techniques described in the previously incorporated  
20 references. The separated particles can then undergo additional  
screening for surface markers which are indicative of one or more  
disease states.

#### EXAMPLE VIII

25

The procedures of Example VII are repeated, except that the cells  
are separated from the magnetic particles and cultured with an  
immortal cell line in accordance with the procedures of Kohler  
30 and Milstein, Nature, Vol. 256, 495-497 (1975). The clones  
produced in this fashion are screened for the production of  
antiserum specific for the surface marker of interest.

EXAMPLE IX

5

The procedures of Example V are repeated, except for the substitution of 0.1% (v/v) citric acid for the acetic acid in the lytic reagent system. This substitution achieved results comparable to that attained in Example V.

10

EXAMPLE X

15 The procedures of Example V are repeated, except for the substitution of 0.1% (v/v) succinic acid for the acetic acid in the lytic reagent system. This substitution achieved results comparable to that attained in Example V.

20

EXAMPLE XI

25 The procedures of Example V are repeated, except for the substitution of 0.1% (v/v) lactic acid for the acetic acid in the lytic reagent system. This substitution achieved results comparable to that attained in Example V.

30

EXAMPLE XII

The procedures of Example V are repeated, except for the substitution of 0.05% (v/v) sulfuric acid for the acetic acid in the

lytic reagent system. This substitution achieved results comparable to that attained in Example V.

- 5 The foregoing Detailed Description and Examples are intended as illustrative of some of the preferred embodiments of the lytic reagents, reagent systems and methods of this invention. It is not the intent of the inventors that these specific embodiments of their invention be interpreted as indicative of the scope or  
10 breadth thereof, but rather simply supportive of the claims which are set forth hereinafter.
-

CLAIMS

1. A lytic reagent system for chemical treatment of a whole blood  
5 sample so as to effect the partitioning of said sample into two  
distinct fractions, an essentially intact leukocyte fraction and a  
lysed erythrocyte fraction, said system comprising a first aqueous  
solution having a lytic reagent comprising a water soluble  
10 compound which at least partially dissociates in aqueous media so  
as to generate free protons and counterions,

said system being characterized in that said first  
aqueous solution comprises a differentiation effective  
15 amount of a lytic reagent and that said system includes a  
second aqueous solution comprising a quench specific for  
said lytic reagent,

said differentiation effective amount of said lytic  
20 reagent, when added to the whole blood sample, effecting:  
(i) a decrease in the pH of the sample from its physiological  
level to a pH in the range of from about 2.6 to about 4.0  
while maintaining the osmolality of the sample at less than  
about 100 mOs; (ii) rapid and essentially complete  
25 hemolysis of said erythrocyte fraction and; (iii) subtle  
changes in said leukocyte fraction to enhance the ability of  
instrumentation to perform differential analysis and  
identification of at least five (5) sub-populations of  
leukocytes; said subtle changes being effected while  
30 preserving said leukocyte fraction in its essentially native  
physiological and/or immunochemical state; and,

said second aqueous solution comprising an alkaline  
salt solution, which upon contact with said lytic reagent  
35 effectively and rapidly arrests the chemical action of said

lytic reagent upon the sample and restores the native physiological environment by stabilizing the lysed sample at a pH in the range of from about 6 to about 7.5 and an osmolality in the range from about 300 to about 330 mOs, so as to permit the analysis of the leukocyte fraction in its native or near native condition.

2. The lytic reagent system of claim 1, characterized wherein said lytic reagent has the following formula



wherein R is H; an aliphatic hydrocarbon radical having from 1-3 carbon atoms; a carbonyl substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; a hydroxy substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; or an aliphatic hydrocarbon radical having from 1-3 carbon atoms and multiple carbonyl and/or hydroxy substituents.

3. The lytic reagent system of claims 1 or 2, characterized wherein said lytic reagent is selected from the group consisting of formic acid; acetic acid; citric acid; succinic acid; lactic acid; or, their respective mixtures.

4. The lytic reagent system of claims 1 or 2, characterized wherein said concentration of lytic reagent in said first aqueous solution, can range from about 0.01 to about 1.0% (v/v).

5. The lytic reagent system of claims 1 or 2, characterized wherein said concentration of lytic reagent in said first aqueous solution can range from about 0.05 to about 0.5% (v/v).

5

6. The lytic reagent system of any one of claims 1, 2, 3, 4 or 5, characterized wherein said first aqueous solution is further characterized as having an additional active component, said additional active component comprising saponin and being  
10 present at an erythrocyte stroma clarification effective amount in the range of from about 0.05 to about 0.20% (w/v).

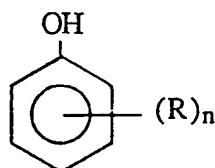
7. The lytic reagent system of claim 1, characterized wherein said  
15 lytic reagent has the following formula:



wherein R is hydroxy, an aliphatic hydrocarbon radical of  
20 1-3 carbon atoms, or aryl.

8. The lytic reagent system of claim 7, characterized wherein said lytic reagent is selected from the group consisting of sulfuric acid;  
25 methanesulfonic acid; ethanesulfonic acid; benzenesulfonic acid; p-toluenesulfonic; m-nitrobenzenesulfonic acid; and, their respective mixtures.

30 9. The lytic reagent of claim 1, characterized wherein said lytic reagent has the following formula:



wherein R is an electron withdrawing group such as  
halogen, cyano, nitro, or any combination of said  
electron withdrawing group; and  
n is 1-3.

10. The lytic reagent system of claim 9, characterized wherein  
said lytic reagent selected from the group consisting of para-  
nitrophenol; meta-nitrophenol; ortho-nitrophenol; 2,4-  
dinitrophenol; para-chlorophenol; para-cyanophenol; 1-chloro-  
2,4-dinitrophenol; and, their respective mixtures.

15

11. A method for partitioning a whole blood sample into two  
distinct fractions, an essentially intact leukocyte fraction and a  
lysed erythrocyte fraction, said method being characterized by  
the following steps performed in the following order:

20

(a) providing a lytic reagent system having:

(i) a first aqueous solution comprising a diluent and a  
lytic reagent comprising a water soluble compound which  
at least partially dissociates in aqueous media so as to  
generate free protons and counterions, said first aqueous  
solution being characterized as containing a  
differentiation effective amount of said lytic reagent,

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said differentiation effective amount of said compound,  
when added to the whole blood sample, effecting: (i) a  
decrease in the pH of the sample from its physiological  
level to a pH in the range of from about 2.6 to about 4.0  
while maintaining the osmolality of the sample at less  
than about 100 mOs; (ii) rapid and essentially complete

35

- 5 hemolysis of the red blood cell fraction and; (iii) subtle changes in the leukocyte cell fraction to enhance the ability of instrumentation to perform differential analysis and identification of at least five (5) sub-populations of leukocytes, said subtle changes being effected while preserving the leukocyte sub-populations in their essentially native physiological and/or immunochemical state; and,
- 10 (ii) a second aqueous solution consisting essentially of an alkaline salt solution of a quenching reagent, which upon contact with the lytic reagent effectively and rapidly arrests the chemical action of said lytic reagent upon the sample and restores the native physiological environment
- 15 by stabilizing the lysed sample at a pH in the range of from about 6 to about 7.5 and an osmolality in the range from about 300 to about 330 mOs, so as to permit the analysis of the leukocyte fraction in its native or near native condition;
- 20 (b) contacting said whole blood sample with said differentiation effective amount of said lytic reagent system;
- 25 (c) allowing said lytic reagent and said sample to interact for an interval not to exceed ten seconds at a temperature in the range of from about 18 to 28°C; and,
- 30 (d) retarding the interaction of said lytic reagent upon said leukocyte fraction of the sample by addition of a quenching reagent, said quenching agent being present in sufficient concentration to essentially immediately retard the lytic activity of the lytic reagent and restore the physiological environment of said leukocyte fraction within the sample.



12. The method of claim 11, characterized by utilizing the lytic reagent system of claim 2.

5

13. The method of claim 11, characterized by utilizing the lytic reagent system of claim 7.

10 14. The method of claim 11, characterized by utilizing the lytic reagent system of claim 9.

15 15. The method of any one of claims 11, 12, 13 or 14, characterized wherein the concentration of said lytic reagent in said first aqueous solution, can range from about 0.05 to about 0.5% (v/v).

20 16. The method of any one of claims 11, 12, 13, 14 or 15, characterized wherein said first aqueous solution is characterized as having an additional active component, said additional active component comprising saponin and being present at an erythrocyte stroma clarification effective amount in the range of  
25 from about 0.05 to about 0.20% (w/v).

30 17. A test kit for partitioning a whole blood sample into two fractions, an essentially intact leukocyte fraction and a lysed erythrocyte fraction, said test kit comprising a first aqueous solution which contains a lytic reagent which at least partially dissociates in aqueous media to generate free protons and counterions,

35

5       said test kit being characterized as having two distinct  
and complementary solutions, said first aqueous solution  
containing a lytic reagent which is effective, when present  
at a differentiation effective amount, to selectively lyse said  
erythrocyte fraction of said whole blood sample and a  
second complementary solution containing a quench,  
specific for said lytic reagent, which is effective when  
present at appropriate amounts, to retard the lytic activity  
of said lytic reagent upon the sample,

10       said differentiation effective amount of said lytic  
reagent, when added to the whole blood sample, effecting:  
(i) a decrease in the pH of the sample from its physiological  
level to a pH in the range of from about 2.6 to about 4.0  
15       while maintaining the osmolality of the sample at less than  
about 100 mOs; (ii) rapid and essentially complete  
hemolysis of said erythrocyte fraction and; (iii) subtle  
changes in said leukocyte fraction to enhance the ability of  
instrumentation to perform differential analysis and  
20       identification of at least five (5) sub-populations of  
leukocytes, said subtle changes being effected while  
preserving said leukocyte fraction in its essentially native  
physiological and/or immunochemical state,

25       a quench consisting essentially of an alkaline aqueous  
salt solution which is effective, upon addition to the sample,  
to rapidly retard the lytic activity of said lytic reagent,  
while stabilizing the lysed sample at a pH in the range of  
from about 6 to about 7.5 and an osmolality in the range of  
30       from about 300 to 330 mOs.

18. The test kit of claim 17, characterized wherein said lytic reagent has the following formula:



5

wherein R is H; an aliphatic hydrocarbon radical having from 1-3 carbon atoms; a carbonyl substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; a hydroxy substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; or an aliphatic hydrocarbon radical having from 1-3 carbon atoms and multiple carbonyl and/or hydroxy substituents.

10

19. The test kit of claims 17 or 18, characterized wherein said lytic reagent is selected from the group consisting of formic acid; acetic acid; citric acid; succinic acid; lactic acid; or, their respective mixtures.

20

20. The test kit of claims 17 or 18, characterized wherein said concentration of lytic reagent in said first aqueous solution, can range from about 0.01 to about 1.0% (v/v).

25

21. The test kit of claims 17 or 18, characterized wherein said concentration of lytic reagent in said first aqueous solution is from about 0.05 to about 0.5% (v/v).

30

22. The test kit of any one of claims 17, 18, 19, 20 or 21, characterized wherein said first aqueous solution is further characterized as having an additional active component, said additional active component comprising saponin and being

present at an erythrocyte stroma clarification effective amount in the range of from about 0.05 to about 0.20% (w/v).

- 5 23. The test kit of claim 17, characterized wherein said lytic reagent has the following formula:



- 10 wherein R is hydroxy, an aliphatic hydrocarbon radical of 1-3 carbon atoms or aryl.

- 15 24. The test kit of claim 23, characterized wherein said lytic reagent is selected from the group consisting of sulfuric acid; methanesulfonic acid; ethanesulfonic acid; benzenesulfonic acid; p-toluenesulfonic; m-nitrobenzenesulfonic acid; and, their respective mixtures.

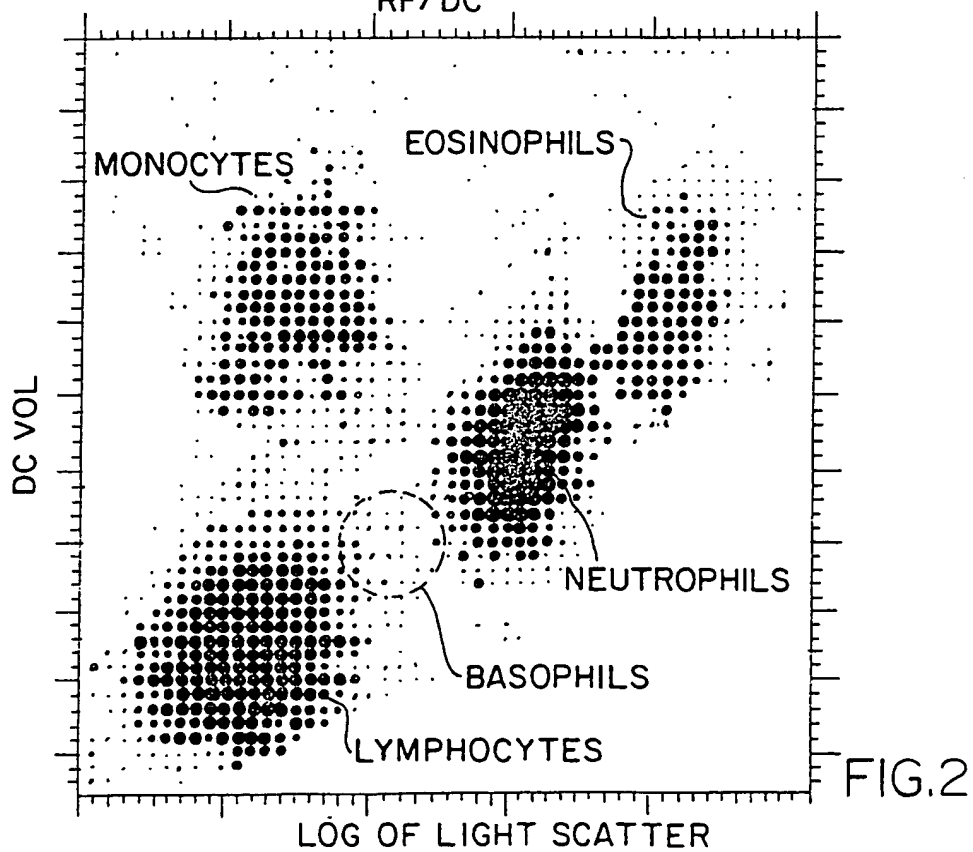
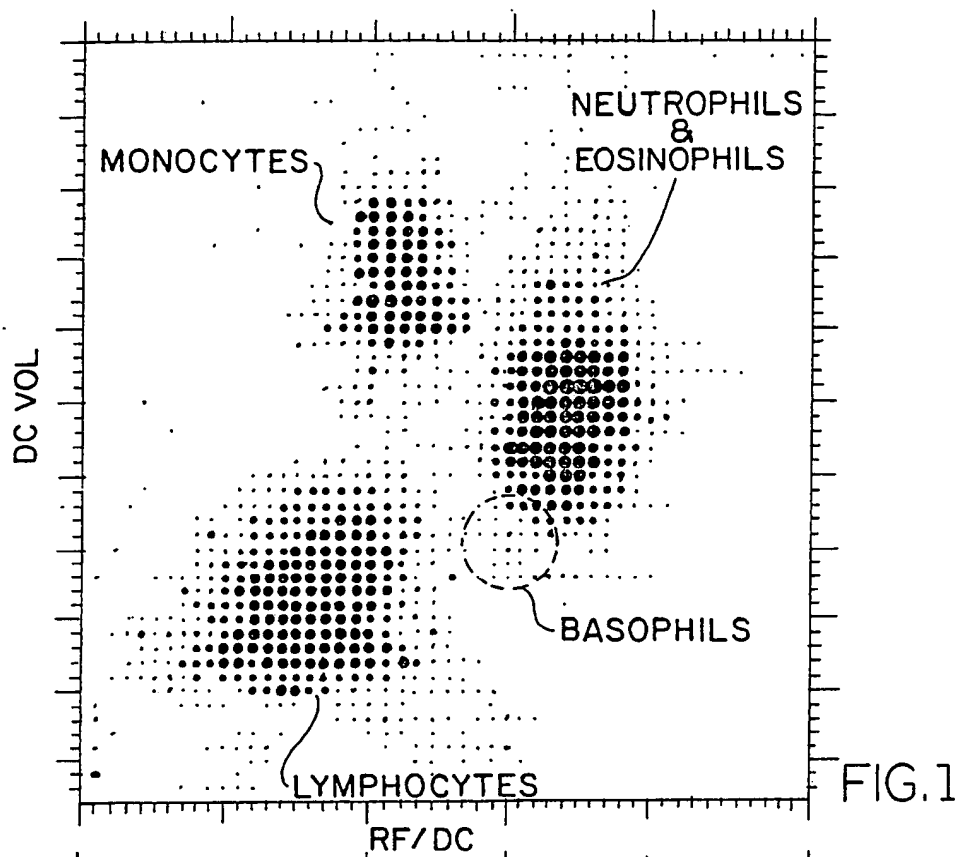
- 20 25. The test kit of claim 17, characterized wherein said lytic reagent has the following formula:



- 30 wherein R is an electron withdrawing group such as halogen, cyano, nitro or any combination of said electron withdrawing group; and n is 1-3.

- 35 26. The test kit of claim 25, characterized wherein said lytic reagent is selected from the group consisting of para-nitrophenol;

meta-nitrophenol; ortho-nitrophenol; 2,4-dinitrophenol; para-chlorophenol; para-cyanophenol; 1-chloro-2,4-dinitrophenol; and, their respective mixtures.



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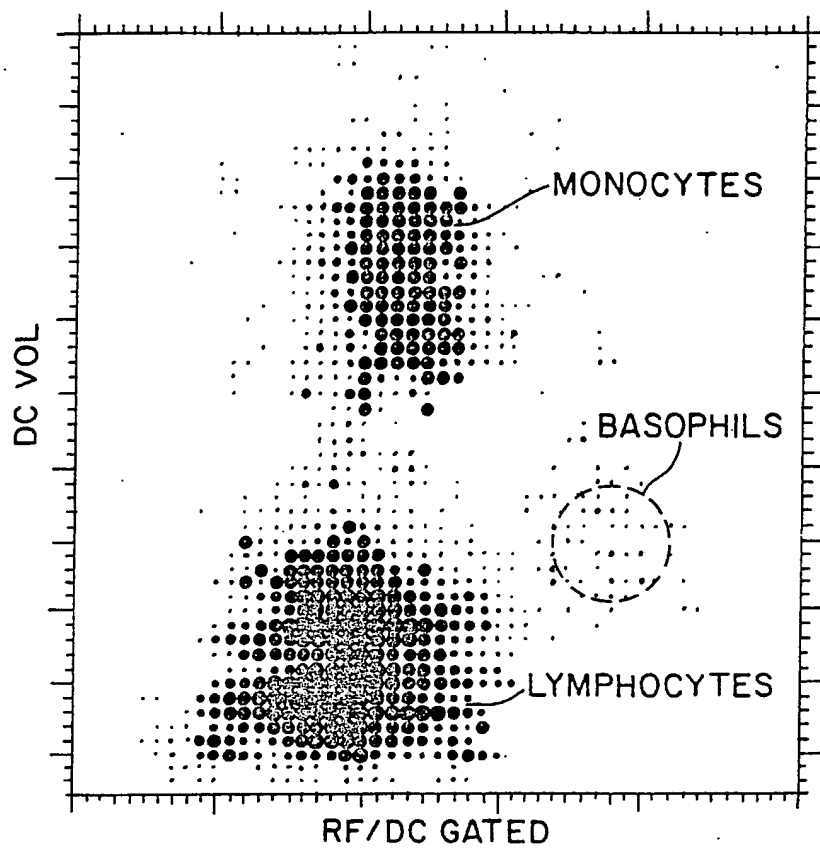


FIG. 3

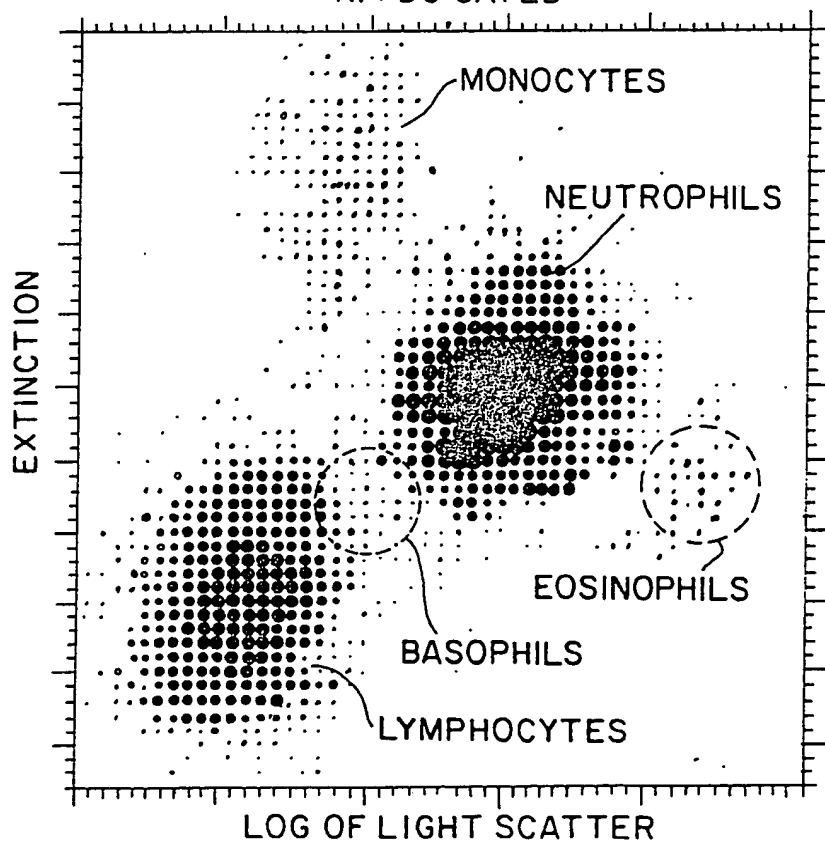


FIG. 4

3/3

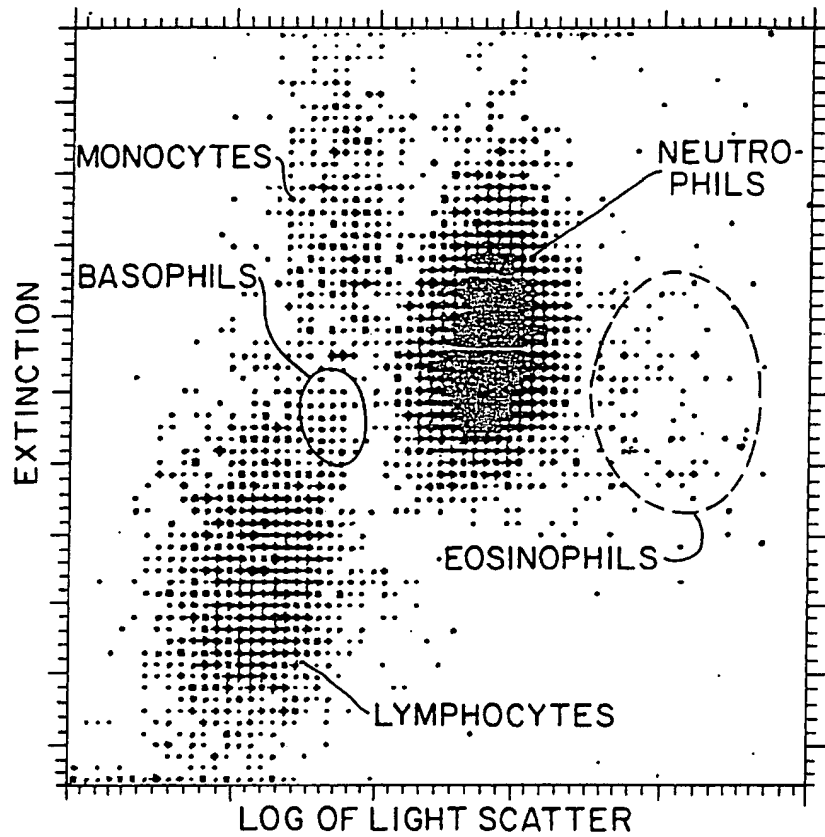


FIG. 5

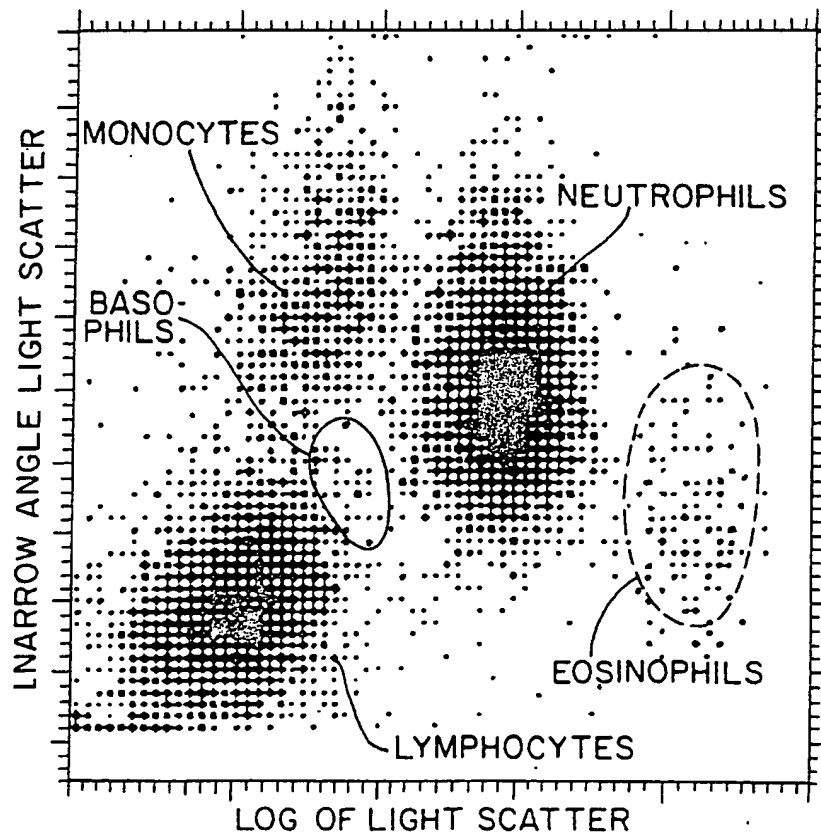


FIG. 6



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/00762

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) -		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(4): G01N 1/28, 33/50		
US. CL.: 422/61; 436/17, 63, 175, 176		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	422/61; 436/8, 10, 17 18, 63, 174, 175, 176, 179	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category *	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>16</sup>
X	CA, A, 1,087,966 (BENVENISTE) 21 October	1-5
Y	1980, see especially page 12, lines 20-29, page 18, lines 10-23, and page 34, lines 20-30.	6, 17-22
Y	US, A, 3,446,751 (WEICHSELBAUM) 27 May 1969, see the entire document.	6, 22
Y	US, A, 4,269,605 (DEAN et al) 26 May 1981 see especially column 6, lines 17-30.	17-22
A	US, A, 3,874,852 (HAMILL) 01 April 1975, see the entire document.	
A	US, A, 4,099,917 (KIM) 11 July 1978, see the entire document.	
A	US, A, 4,250,051 (ARMSTRONG) 10 February 1981, see the entire document.	
A	US, A, 4,286,963 (LEDIS et al) 01 September 1981, see the entire document.	
<p>* Special categories of cited documents: <sup>18</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search :	Date of Mailing of this International Search Report :	
09 June 1983	27 JUN 1988.	
International Searching Authority :	Signature of Authorized Officer <sup>19</sup>	
ISA/US	Robert J. Hill, Jr.	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages <sup>1</sup>	Relevant to Claim No <sup>1</sup>
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- |   |   |  |
|---|---|--|
| A | US, A, 4,324,685 (LOUDERBACK) 13 April 1982, see the entire document.   |  |
| A | US, A, 4,346,018 (CARTER et al) 24 August 1982, see the entire document.  |  |
| A | US, A, 4,436,821 (RYAN) 13 March 1984, see the entire document.   |  |
| A | US, A, 4,485,175 (LEDIS et al) 27 November 1984, see the entire document.   |  |
| A | JP, A, 54-68291 (MIKI ENGINEERING) 06 January 1979, see the abstract.   |  |
| A | J. MIALE, "Laboratory medicine-HEMATOLOGY", third edition, published 1967 by The C. V. Mosby Company (Saint Louis), see page 1122.  |  |
| A | Acta Paediatrica Scandinavica, volume 60, issued 1971, F. Schettini et al, "Acid Lysis of Red Blood Cells in Normal Children", see pages 17-21.   |  |
| A | Transfusion, volume 13, number 5, issued September-October 1973, A. da Costa et al, "Effects of Ethacrynic acid on Human Red Blood Cells", see pages 305-313.   |  |
| A | Zeitschrift fuer Immunologie-Forschungsgemeinschaft, volume 155, issued 1978, U. Rother et al, "Deviated Lysis: Lysis of Unsensitized Cells by Complement. V. Generation of the Activity by Low pH or Low Ionic Strength", see pages 118-129. |  |

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